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Two new records of *Agaricus* from Southwest China

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Abstract—Two specimens of *A. brunneosquamulosus* (from China and Thailand) and four specimens of *A. duplocingulatus* (from China) were identified and studied by morphological and ITS sequence analyses. They are presented here with descriptions, line drawings, and color photographs as new records for China. Our additional molecular sampling of *A. duplocingulatus* has revealed intraspecific variability within this species.

Key words—*Agaricaceae,* rDNA, taxonomy

Introduction

Agaricus L. is a well-known genus containing species with a high nutritional and medicinal value, such as the famous cultivated mushrooms *A. bisporus* (J.E. Lange) Imbach and *A. subrufescens* Peck. Zhao et al. (2011) recognized 386 species in this cosmopolitan genus, a number that has subsequently risen to over 400 with the discovery of numerous novel taxa from Asia, Australia, and Europe (Chen et al. 2012, Gui et al. 2015, Lebel 2013, Lebel & Syme 2012, Li et al. 2014, Parra 2013, Zhao et al. 2012). Eight sections were traditionally recognized in the genus, long classified based on temperate species (Challen et al. 2003; Kerrigan et al. 2005; Kerrigan et al. 2008; Parra 2008, 2013). However, following a phylogenetic study of samples from both temperate and tropical regions by Zhao et al. (2011), eleven new lineages comprising tropical or subtropical taxa were revealed.

One new clade determined by Zhao et al. (2011) has been phylogenetically reconstructed and identified as corresponding with *Agaricus* sect. *Brunneopicti* Heinem. (Chen et al. 2015). The section has been delimited to accommodate species that combine the following morphological characters: punctiform squamulose remnants of the universal veil or brownish larger squamules on pileus surface; a double or complex annulus with cortinate fibrils on the under side; context when bruised discoloring faint to strong yellow, orange, rufescent, brownish rufescent, or red; odor of bitter almond, phenol, or solvent; a positive KOH reaction, and a negative or (rarely) weakly positive Schäffer's reaction (Chen et al. 2015). The section, which has a strictly palaeotropical distribution, contains 16 species.

Southwestern China is known as home to glacial refugia and plays an important role in postglacial biological recolonization. In order to reveal the species spreading patterns and biodiversity of *Agaricus*, since 2010 we have conducted a series of investigations in this area during the July–August rainy season. As one result, two representatives of *A.* sect. *Brunneopicti*—*Agaricus brunneosquamulosus* and *A. duplocingulatus*—are newly recorded from China.

Materials & methods

Morphological study

Specimens were collected in Yunnan Province, China, during the rainy season. Photographs were taken immediately in the field. Each specimen was wrapped in aluminum foil or kept separate in a plastic collection box. Macroscopic characters were recorded and chemical tests were performed immediately after the specimens reached the laboratory according to Largent (1986). The specimens were dried completely at 70°C in a food drier, sealed in plastic bags, and deposited in the herbarium of Southwest Forestry University, Kunming, China (SWFC), with duplicates in Herbarium, Mycologicum Academiae Sinicae, Beijing, China (HMAS). An additional specimen from Thailand (deposited in HMAS) was included in the study.

Following the methodology described by Largent (1986), microscopical features (basidiospores, basidia, cystidia, pileipellis, annulus hyphae) of each dried specimen were examined. At least 20 measurements were made to calculate the dimensions presented as follows: X = the means ± SDs of length × width, Q = basidiospore length : width ratio, and Q_m = the mean ± SD of Q values.

DNA extraction, PCR, and sequencing

DNA was extracted from the dried fruiting bodies using the E.Z.N.A. Forensic DNA Extraction Kit (D3591-01, Omega Bio-Tek). The internal transcribed spacer (ITS) regions were amplified with the primers ITS4 and ITS5 following the protocol of White et al. (1990). DNA sequencing was performed in commercial biotechnology company.

TABLE 1. *Agaricus* specimens included in the phylogenetic analyses.

SPECIES	SAMPLE	ORIGIN	ITS SEQUENCE
A. sp. 1	C3182	Togo	KJ540955
A. sp. 2	LD2011027	Thailand	KJ540955
A. sp. 3	ZRLwxh3115	China	KR812336
A. bingensis	ADK1992	Bénin	KJ540954
	C3155	Togo	KJ540950
A. niveogranulatus	LD2011025	Thailand	KJ540957
	LD2011023	Thailand	KJ540958
	LD2011024	Thailand	KJ540959
	PYP009	Thailand	KJ540960
A. chiangmaiensis	NTS113	Thailand	JF514513
A. brunneopunctatus	ADK2564	Bénin	JF514518
A. toluenolens	CA911	Thailand	KJ540947
	CA926	Thailand	KJ540948
A. sp. 4	NTT117	Thailand	JF514534
A. sordidocarpus	LD201237	Thailand	KJ540946
A. subsaharianus	ADK4732	Burkina-Faso	JF440300
A. sp. 5	CA800	Thailand	JF727826
A. megacysidiatus	LD2012168	Thailand	KF305947
	LD2012179	Thailand	KF305946
	NTS115	Thailand	KC971098
	NTS116	Thailand	JF514532
A. sp. 6	NT019	Thailand	JF727844
A. brunneosquamulosus	NTT118	Thailand	KJ540970
	LD201238	Thailand	KJ540969
	ZRL3005	Thailand	KR869107
	ZRL4017	Thailand	JF691549
	LD2012105	Thailand	KJ540968
	ZRL133	Thailand	KR812337
	ZRL2013266	China	KR812340
A. cf. *inoxydabilis*	LAPAF1	Togo	JF727841
A. duplocingulatus	ZRL3064	Thailand	KR869108
	CA903	Thailand	KJ540962
	LD2012177	Thailand	KJ540961
	LD201218	Thailand	KJ540967
	LD201233	Thailand	KJ540965
	LD201274	Thailand	KJ540963
	LD201275	Thailand	KJ540964
	ZRL3031	Thailand	JF691550
	ZRL3041	Thailand	KR869109
	NTT34	Thailand	JF514536
	ZRL2013328	China	KR812341
	ZRL2012267	China	KR812339
	ZRL2012120	China	KR812338
	ZRL2013348	China	KR812342

Phylogenetic analyses

Our original sequences plus those retrieved from GenBank (Table.1) were first aligned using ClustalX 2.0 (Thompson et al. 1997) and then manually adjusted in BioEdit v.7.0.4 (Hall 2007). Bayesian analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). One million generations using a GTR+I+G model nucleotide substitution detected by MrModeltest 2.2 (Nylander 2004) were run for six Markov chains and sampled every 100th generation resulting in 10,000 trees. Maximum Parsimony analysis was performed using PAUP*4.0b10 (Swofford 2004). One thousand heuristic searches were conducted with random sequence addition, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Parsimony bootstrap values were obtained from 1000 bootstrap replicates, with starting trees obtained via stepwise addition, random sequence addition, and max-trees set to 1,000,000.

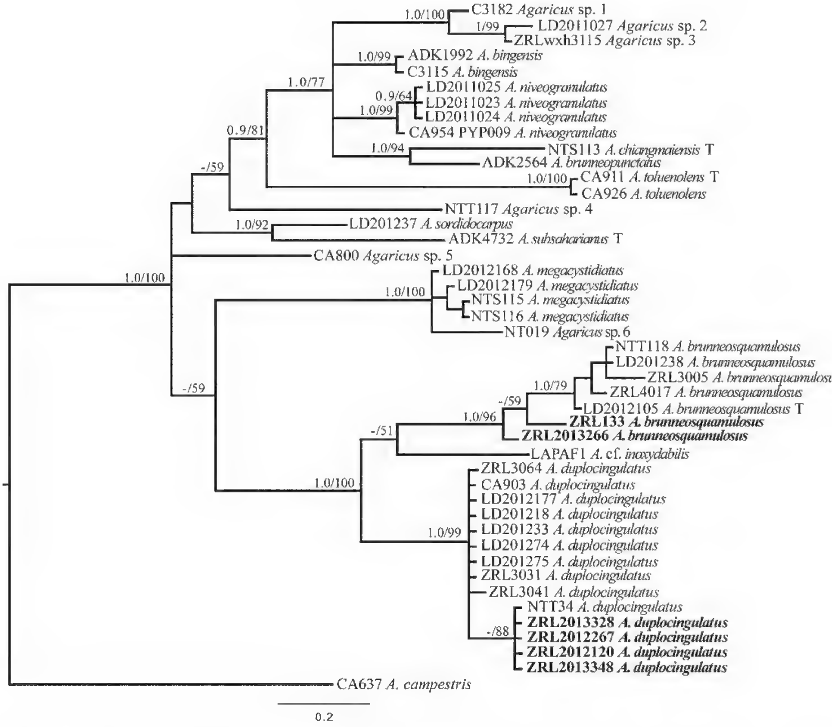
1.0/100
C3182 *Agaricus* sp. 1
1/99
LD2011027 *Agaricus* sp. 2
ZRLwxh3115 *Agaricus* sp. 3
1.0/99
ADK1992 *A. bingensis*
C3115 *A. bingensis*
1.0/77
0.9/64
LD2011025 *A. niveogranulatus*
1.0/99
LD2011023 *A. niveogranulatus*
LD2011024 *A. niveogranulatus*
CA954 PYP009 *A. niveogranulatus*
0.9/81
1.0/94
NTS113 *A. chiangmaiensis* T
ADK2564 *A. brunneopunctatus*
-/59
1.0/100
CA911 *A. toluenolens* T
CA926 *A. toluenolens*
NTT117 *Agaricus* sp. 4
1.0/92
LD201237 *A. sordidocarpus*
ADK4732 *A. subsaharianus* T
CA800 *Agaricus* sp. 5
1.0/100
LD2012168 *A. megacystidiatus*
1.0/100
LD2012179 *A. megacystidiatus*
NTS115 *A. megacystidiatus*
NTS116 *A. megacystidiatus*
NT019 *Agaricus* sp. 6
NTT118 *A. brunneosquamulosus*
LD201238 *A. brunneosquamulosus*
ZRL3005 *A. brunneosquamulosus*
-/59
1.0/79
ZRL4017 *A. brunneosquamulosus*
-/59
LD2012105 *A. brunneosquamulosus* T
1.0/96
ZRL133 *A. brunneosquamulosus*
-/51
ZRL2013266 *A. brunneosquamulosus*
LAPAF1 *A.* cf. *inoxydabilis*
ZRL3064 *A. duplocingulatus*
1.0/100
CA903 *A. duplocingulatus*
LD2012177 *A. duplocingulatus*
LD201218 *A. duplocingulatus*
1.0/99
LD201233 *A. duplocingulatus*
LD201274 *A. duplocingulatus*
LD201275 *A. duplocingulatus*
ZRL3031 *A. duplocingulatus*
ZRL3041 *A. duplocingulatus*
NTT34 *A. duplocingulatus*
ZRL2013328 *A. duplocingulatus*
-/88
ZRL2012267 *A. duplocingulatus*
ZRL2012120 *A. duplocingulatus*
ZRL2013348 *A. duplocingulatus*
CA637 *A. campestris*
0.2

Figure 1. Phylogeny of *Agaricus* sect. *Brunneopicti* generated from MrBayes analysis of ITS sequences rooted with *A. campestris*. New sequences are set in bold font. Bayesian posterior probability (PP) values >0.9, and parsimony bootstrap support (BS) values >50% are given at the nodes.

Results

Phylogenetics analyses

The dataset comprised 45 sequences representing 17 species with *A. campestris* as outgroup (Zhao et al. 2011). The final alignment comprised a total of 663 characters, of which 517 were constant, 39 parsimony-uninformative, and 107 parsimony-informative. The phylogenetic trees generated by MP and Bayesian methods exhibited very similar topologies, with the Bayesian tree shown in Fig.1. Collections ZRL2013266 (from China) and ZRL133 (from Thailand) clustered in the *A. brunneosquamulosus* clade with strong support (1/96); and Chinese collections ZRL2013328, ZRL2012267, ZRL2012120, and ZRL2013348 grouped with NTT34 (from Thailand; Zhao et al. 2011) in the *A. duplocingulatus* clade with strong support (1/99).

Taxonomy

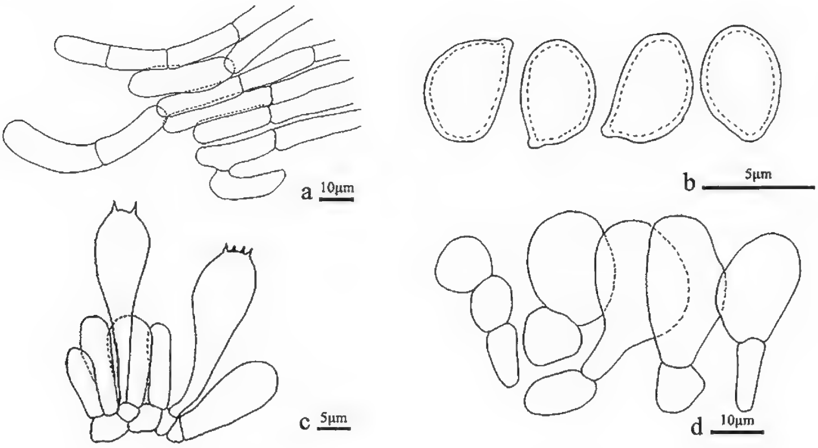

Figure 2. *Agaricus brunneosquamulosus* (ZRL2013266):
a. Pileipellis; b. Basidiospores; c. Basidia; d. Cheilocystidia.

Agaricus brunneosquamulosus L.J. Chen, R.L. Zhao, K D. Hyde & Callac,
Phytotaxa 192(3): 154 (2015). Figs 2, 4A,B

Macroscopical characters: Pileus 26 mm in diam, parabolic, convex, margin decurved; surface dry, with appressed triangular fibrillose squamules, light brown to brown against white background; Lamellae free, crowded, pink to brown, 3 mm broad, with 3 series of lamellulae. Stipe 60 × 8–16 mm (at base), thickening towards the base, with long rhizomorphs; surface white, covered by fine fibrils; context narrow, hollow. Annulus double (two adhering layers): upper layer a membranous remnant of the partial veil, rigid, pendant, white,

≤21 mm diam, 3 mm thick; bottom layer a fibrillose-membranous remnant of the universal veil, fragile; white above and with brown fibrils below. CONTEXT 3 mm thick, white; discoloration indistinct when touched, none when cut. Odor not noted.

Macrochemical reactions: KOH—slightly yellow. Schäffer's reaction—negative.

Microscopical characters: BASIDIOSPORES 4.1–5.6 × 2.8–3.7 µm, [X = 4.8 ± 0.4 × 3.3 ± 0.3 µm, Q = 1.21–1.65, Q_m = 1.47 ± 0.13, n = 20], ellipsoid, smooth, brown, thick-walled. BASIDIA 4-spored, 18.5–30.6 × 5.9–8.7 µm, narrowly clavate to clavate, hyaline, smooth. CHEILOCYSTIDIA 14.6–27.6 × 10.9–25.3 µm, pyriform, clavate to broadly clavate, basally septate or catenulate with 2–3 ellipsoid to subspherical cells in chain, hyaline, smooth. PLEUROCYSTIDIA absent. PILEIPELLIS a cutis, hyphae 4.3–11.5 µm diam, cylindrical, light brown, smooth, sometimes slightly constricted at the septa.

HABIT: Solitary in forest.

SPECIMENS EXAMINED: **CHINA**, YUNNAN PROVINCE, Teng Chong County, Laifeng Mountain, 18 July 2013, Zhou Junliang, ZRL2013266 (HMAS, SWFC; GenBank KR812340). **THAILAND**, CHIANG MAI PROVINCE, Mae Taeng District, Ban Pha Deng Village, Mushroom Research Centre, 15 May 2008, Zhao Ruilin, ZRL133 (HMAS; GenBank KR812337).

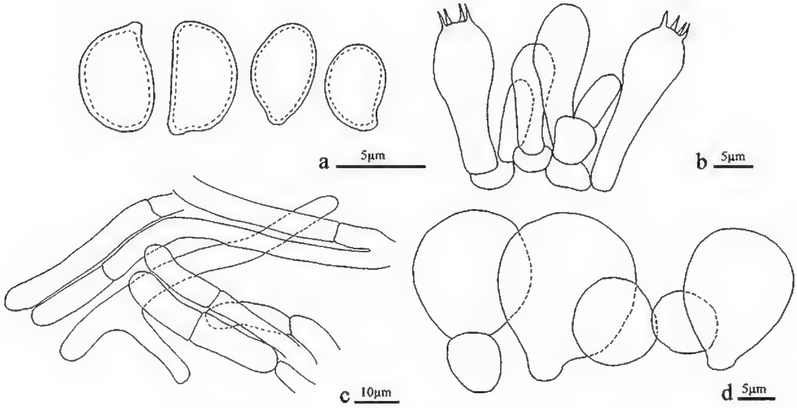

FIGURE 3. *Agaricus duplocingulatus* (ZRL2013328):
a. Basidiospore; b. Basidia; c. Pileipellis; d. Cheilocystidia.

Agaricus duplocingulatus Heinem., Bull. Jard. Bot. Natl. Belg. 50: 32 (1980). FIGS 3, 4C–H.

Macroscopical characters: PILEUS 27–87 mm diam, convex, margin decurved when young, becoming straight when mature; surface dry, covered

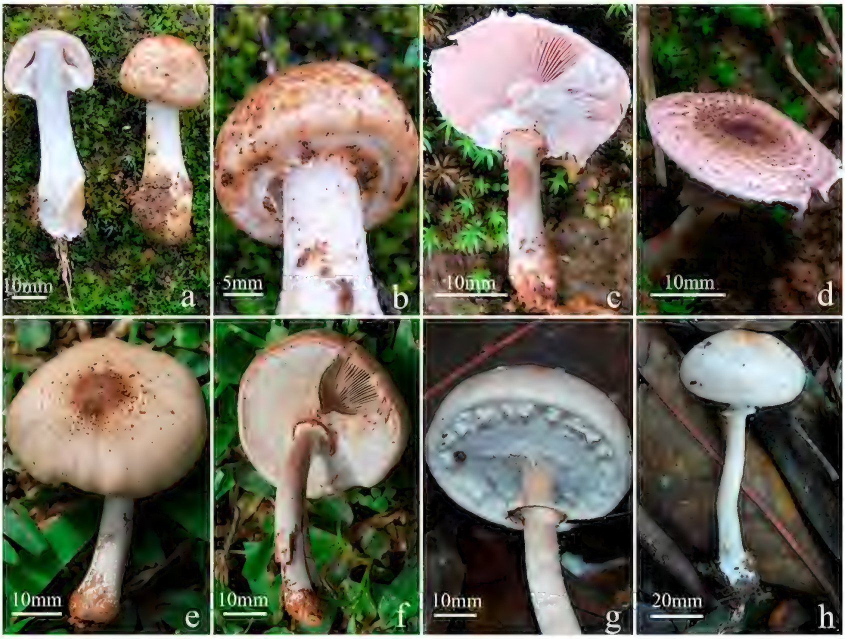

Figure 4. ***A. brunneosquamulosus*** (a, b: ZRL2013266). Habit.
A. duplocingulatus (c, d: ZRL2012120; e, f: ZRL2012267; g, h: ZRL2013328). Habit.

with reddish brown appressed fibrillose squamules on a white background; squamules congregated on the disc and concentrically arranged elsewhere; fibrillose or mud-cracked [areolate?]; surface becoming dull red when wet. Lamellae free, crowded, lamellulae in 5 tiers, 1–6 mm broad, pink when young, brown when mature. Stipe 30–43 × 4–9 mm, cylindrical with enlarged (4–23 mm diam) base, with rhizomorphs, hollow; surface silky; white, turning reddish brown when bruised or cut. Annulus double, two separate layers: upper layer wider (47 mm diam), persistent, membranous, white, smooth above and fibrillose to floccose below; lower layer, narrower (12 mm diam), bracelet-like, fugacious, movable, white and floccose above, smooth with a brown margin below. Context 4 mm broad, firm; discoloration variable: distinctly yellow when touched and red when cut or no discoloration when touched or cut. Odor almond-like.

Macrochemical reactions: KOH reaction yellow. Schäffer's reaction negative.

Microscopical characters: Basidiospores 4.5–6.5 × 3.6–4.5 µm [X = 5.7 ± 0.3 × 4.0 ± 0.2 µm, Q = 1.30–1.7, Q_m = 1.40 ± 0.10, n = 20], ellipsoid, smooth, brown, thick-walled. Basidia 4-spored, 14.5–19.3 × 4.9–7.5 µm, clavate, hyaline, smooth. CHEILOCYSTIDIA 14.2–27.3 × 8.1–19.6 µm, globular to pyriform, hyaline, smooth. PLEUROCYSTIDIA absent. PILEIPELLIS a cutis, hyphae 2.3–7.0 µm diam, cylindrical, light brown, smooth, slightly constricted at the septa.

HABIT: Solitary in forest.

SPECIMENS EXAMINED: **CHINA**, YUNNAN PROVINCE, Ying Jiang County, Tong Biguan National Natural Reserve, 19 July 2013, Yu Qinghua, ZRL2013328 (HMAS, SWFC; GenBank KR812341); 20 July 2013, Yu Qinghua, ZRL2013348 (HMAS, SWFC; GenBank KR812342); Cang Yuan county, Nan Gunhe National Natural Reserve, 7 July 2012, Zhao Ruilin, ZRL2012120 (HMAS, SWFC; GenBank KR812338); An Kang Village, 11 July 2012, Philippe Callac, ZRL2012267 (HMAS, SWFC; GenBank KR812339).

Discussion

Agaricus brunneosquamulosus, originally described from Thailand, is characterized by small sporocarps, tiny ferruginous brown appressed triangular squamules on the pileus surface, and a complex annulus with woolly scales and cortinate fibrils on its lower surface (Chen et al. 2015). The Chinese material generally agrees with original description, except for the uncertain annulus morphology of annulus and occasional presence of short catenulate cheilocystidia. These annular and cheilocystidial differences may be due to the immaturity of the Chinese collection. The ITS sequence alignment does indeed show a five nucleotide difference between the Thai and Chinese collections; however, all the differences are transition mutations (T/C or A/G), polymorphisms commonly observed in intraspecific variation (Zhang et al. 2009, Yadav 2014). In the absence of significant morphological differences between the Thai and Chinese material, we for the time recognize the Chinese material as *A. brunneosquamulosus*. Collection of more young to mature materials in near future will undoubtedly refine our determination.

Agaricus duplocingulatus, first described from Singapore and recently reported from Thailand, is characterized by brown ferruginous appressed squamules on the pileus surface, a double annulus with the lower one movable, an ochraceous to reddish discoloration when cut or bruised, and catenulate cheilocystidia (Heinemann 1980, Chen et al. 2015). The four Chinese collections, which generally match the species circumscription, are more variable in sporocarp size (pileus 27–87 mm diam). Comparison of the ITS sequences reveals nucleotides that differ at four polymorphic positions in more than 16 positions, also by Chen et al. (2015). This degree of intraspecific variability is rarely observed in *Agaricus,* and multi-gene analysis may be helpful in determining whether *A. duplocingulatus* represents a complex of species. Presently, we adopt the identification by Chen et al. (2015).

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